中華農業研究(J. Agric. Res. China) 52:324~333(2003)

Method Development and Application for Rapid Detection of the Anticholinergic Residues in Rice[1]

Ching-hua Kao[2, 3], Chia-lin Tzeng[2] and Edward Y. Cheng[2]

Abstract

Kao, C. H. C. L. Tzeng and Edward Y. Cheng. 2003. Method development and application for rapid detection of the anticholinergic residues in rice. J. Agric. Res. China 52:324-333.

Among several pesticides registered for the protection of rice crop in Taiwan, more than 40 organophosphorus and carbamate insecticides have caused concern due to their residues in harvested rice. Although chemical analysis of residues has been applied to rice, a rapid method to detect harmful levels of pesticide residues will be helpful before residue-contaminated rice is stored, processed or marketed. The residues most commonly detected in rice are those of anticholinergic agents such as, methamidophos, acephate, ethion, pirimiphos-methyl, carbofuran, carbaryl, methomyl, and BPMC. A rapid and highly sensitive acetylcholinesterase test, the rapid bioassay of pesticide residues (RBPR), that has currently been used to detect residues of organophosphate and carbamate insecticides in fruits and vegetables, was modified and tested on three groups of rice samples with known quantities of pesticide residues. Twenty-five samples, classified as group I, had no pesticide residues; 28 samples, classified as group II, contained organophosphorus and carbamate residues under the tolerance level; and 6 samples, classified as group III, contained residues of above-mentioned insecticides above tolerance level. All 59 samples, extracted by whole grain extraction or freshly ground powder extraction, were tested for their anticholinergic activity. The whole grain extraction showed group I samples to have average acetylcholinesterase inhibition of merely 6.7 %; group II, 27.6%; while group III, 60.2%. The 95% confidence range of the means of group I, II, and III were statistically separable and ranged from 1.8-11.6%, 20.0-35.3%, and 41.2-79.1%, respectively. The acetylcholinesterase inhibition correlated positively with the chemical analysis of organophosphorus and carbamate residues. In extraction of ground rice, two samples in group III varied considerably resulting in overlapping of 95% confidence ranges of group II and III. The study indicated that the acetylcholinesterase test is sensitive enough to detect the residues of insecticide in rice without interference of plant chemicals in rice, and it is quantitatively correlated to chemical analysis of insecticide residues. Between two extraction methods, the whole grain treatment is preferred over rice powder method. In a hypothetic analysis of 59 samples, with detection threshold set at 60%, 50%, and 40 % acetylcholinesterase

inhibition, the correct detection rate for violation were 50.0%, 66.7%, and 88.3%, respectively. The detection of false negatives, decreased to 5.5%, 3.9%, and 2.1%, while unnecessary rejection rate increased from 40%, 50%, and 58%, respectively. The acetylcholinesterase inhibition test can be used effectively in (1) verification of organic rice, (2) comparison of safety of rice from different regions, seasons and year, (3) providing indexes of total toxicity of organophosphorus and carbamate insecticide residues to fit the requirement of FQPA. The reliability, economy, time saving, and user-friendly characteristics of RBPR will facilitate screening of larger number of rice samples within a very short period for gathering basic safety information.

Key words : RBPR, Rice, Organophosphorus insecticides, Carbamate insecticides, Acetylcholinesterase.

INTRODUCTION

The anticholinergic test can be used to screen fruits and vegetables for contamination of organophosphours (OP) and carbamate (Carb) insecticide residues (Voss *et al.* 1971). Based on the inhibition of acetylcholinesterase (AChE) by these insecticides, a test utilizing highly sensitive housefly AChE has been developed for rapid screening of fruits and vegetables for pesticide residues. This residue safeguard system, named RBPR (Rapid Bioassay of Pesticide Residues), designed by Taiwan Agricultural Research Institute (TARI) (Chiu *et al.* 1991) has been used at agricultural extension level throughout Taiwan. RBPR is widely accepted as the residue control measure for agricultural commodities in Taiwan. Farmers' associations, wholesale markets, and food suppliers to military and schools have established more than 120 RBPR stations to inspect fruits and vegetables for pesticide residues. Some of these stations have been operating for over 15 years and are gradually becoming integrated into the marketing infrastructure.

Although RBPR has never been applied to cereals, utilization of this method is theoretically feasible. Rice is a staple food in the Orient, including Taiwan. Currently, only chemical analysis is applied to monitor the pesticide residues in rice. Due to the fact that analysis is laborious, expensive, and time consuming, pesticide residue problems, if they exist, get reported months after harvesting, when rice had already been mixed, processed, and even marketed. Hence, a rapid residue detection system is needed to improve the residue control in rice.

More than 40 insecticides belonging to OP and Carb groups are registered on rice (TACTRI 2001). These chemicals can easily be detected by the RBPR tests. Recently, serious concern has been raised on residues of OP and Carb insecticides because of their neurotoxicity (US EPA 2000a; 2000b). United States Food Quality Protection Act (FQPA) passed in 1996 (US Congress 1996) emphasizes the need to group together residues of pesticides with common toxic mechanism for safety consideration (US EPA 2002a; 2002b). In this respect the US Environmental Protection Agency has placed the priority of OP and Carb insecticides due to their common mechanism of action on AChE (US EPA 1999). We, therefore, attempted to study whether residues of OP and Carb in rice can be detected by the anticholinergic test.

1. Contribution No.2173 from Agricultural Research Institute, Council of Agriculture. Accepted : October 1, 2003.

2. Entomologist, Project Assistant and Senior Entomologist, respectively, Applied Division Zoology, ARI, Wufeng, Taichung, Taiwan, ROC.

3. Correspondence author, e-mail: chkao@wufeng.tari.gov.tw ; Fax: (04)23309097.

MATERIALS AND METHODS

Sample sources

Field samples from freshly harvested rice crop from normal production system were collected. A total of 59 samples with known quantity of OP and Carb insecticide residues were used for RBPR. The detected insecticides and their current tolerance in rice are: methamidophos (0.1 ppm), acephate (0.5 ppm), ethion (0 ppm), pirimiphos-methyl (1.0 ppm), carbofuran (0.5 ppm), carbaryl (0.5ppm), methomyl (0.5 ppm), and BPMC (0.5 ppm). Based on the quantity of residue detected, these samples were grouped into three categories. Twenty-five samples were classified as group I with no detectable residues, 28 samples with residues under the tolerance limit were classified as group II, and the remaining 6 samples containing residues over tolerance limit were placed in group III.

Enzyme and other chemicals used

The AChE was purified from the head of housefly, *Musca domestica* L., reared in insecticide-free environment at TARI since 1950s. The lyophilized powder of the enzyme was stored at -20℃ (Chiu *et al.* 1991), and the specificity activity of AChE used is 1.84 μmoles ATCI/min/mg protein. The chemicals used in determination of AChE inhibition reaction, the Ellman test (Ellman *et al.* 1961)-DTNB(5, 5-dithio-bis-2-nitrobenzoic acid), Na_2HPO_4, and NaH_2PO_4 -were purchased from Merck, and ATCI (acetylthiocholine iodide) from Sigma.

Sample extraction and processing

The rice samples were extracted by two different methods. In the first, the whole rice grains were soaked in PBS buffer (pH 8.0) under ultrasonic treatment for 20 min before the AChE inhibition assay (Fig. 1). In the second, the whole rice grains were milled into fine powder and soaked in PBS buffer under ultrasonic treatment for 20 min before the assay. For each sample, 1.6-1.7 g of rice grains was used. Each assay had 4 replicates.

Instruments used

The ultrasonic treatment was performed in a Branson 8210 vessel. The color development in the assay of AChE inhibition was measured on ELISA reader (SpectraMAX 340, Molecular Devices) at the wavelength of 412 nm (Cheng *et al.* 2000).

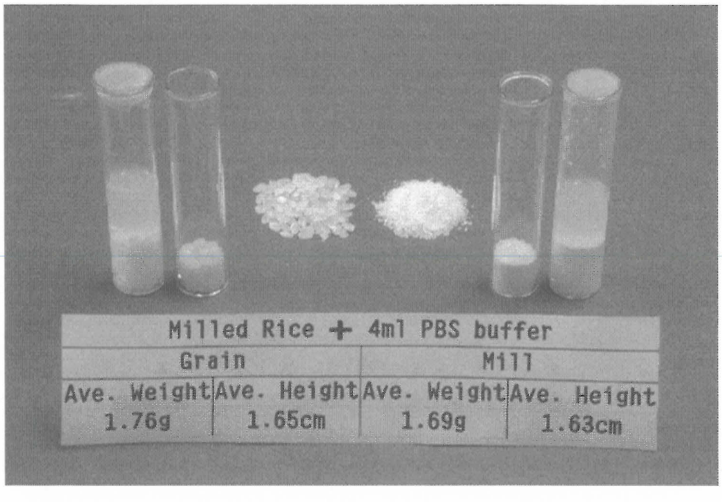

Fig. 1. Different extraction methods for determining the acetylcholinesterase inhibition.

Testing procedures

PBS buffer (0.1 M, pH 8.0), AChE solution (3 mg in 1 ml distilled water), diluted DTNB, and ATCI mixture (0.4 mg DTNB + 0.4 mg ATCI in 1 ml distilled water) were used. Four milliliters of PBS buffer, 1.7 g rice, and 40 µl AChE solution were incubated under ultrasonic treatment for 20 min. An aliquot of 100 µl of the extract was transferred into reading well of microplate and 50 µl of DTNB and ATCI mixture was added and the color developed was read on ELISA Reader (Cheng *et al.* 2000).

TLC confirmation

All rice samples with positive AChE inhibition were subjected to thin layer chromatography (TLC) separation for identification of anticholinergic source. The details of TLC procedure are described in RBPR Working Manual (Cheng *et al.* 1999). The three developing solvent systems used are: (1) 2 parts of methylene chloride + 1 part of ethyl acetate; (2) 7 parts of n-hexane + 3 parts of acetone; and (3) 4 parts of water + 25 parts of methanol + 65 parts of chloroform. The Rf values of chemicals in suspected samples were compared with the Rf values of the probable insecticides (Gänshirt 1966). Methamidophos and acephate were used as standards for comparison of Rf values.

RESULTS

The results of AChE inhibition test of 59 rice samples by two different extraction methods are summarized in Table 1. In the whole grain extraction, 8 samples of the group I showed mild enzyme inhibition, while the remaining 17 samples showed no inhibition at all. The average enzyme inhibition for the whole group, 6.72%, is considered low. Among 28 samples in group II, 25 had positive AChE inhibition, while 3 samples showed no inhibition. The average enzyme inhibition was 27.64%. The group III samples, all of which violated tolerance limit, showed strong anticholinergic activity in all of them with average enzyme inhibition of 60.17%. The 95% confidence ranges of AChE inhibition are 1.84~11.6%, 19.99~35.29%, and 41.24~79.10%, respectively, for group I, II, and III (Table 1). The statistical separation of the confidence ranges is significant without any overlapping.

In milled rice extraction, among 25 samples in group I, 13 showed no AChE inhibition, and 12 had mild inhibition. The AChE inhibition of the whole group averaged 9.28%. In group II only 2 samples out of 28 failed to show AChE inhibition. The average inhibition for the whole group was 30.07%. All 6 samples in group III showed AChE inhibition with average for the entire group being 53.0%. The enzyme inhibition in 2 samples of group III was reduced after grinding the rice grain, while in the remaining 4 samples, the enzyme inhibition was similar to whole grain. In general, grinding did not affect the enzyme inhibition, however, grinding increased variation and standard error. The confidence range of milled samples was less desirable compared to whole grain extraction, 3.13-15.43%, 22.72-37.42%, and 29.38-76.62%, respectively for group I, II, and III. The reduction in AChE inhibition in group III introduced greater experimental error resulting into overlapping of the 95% confidence range between group II and III, which made them statistically inseparable.

Comparison of sample grouping by AChE inhibition and chemical analysis tests is summarized in Table 2. AChE inhibition strongly correlated with the chemical analysis of the insecticide residue in rice samples. Most of the samples in group I had the lowest enzyme inhibition. Most of the samples in group II had medium enzyme inhibition, ranging from 30 to 60%. All 6 samples in group III had medium to high AChE inhibition.

Table 1. The toxicity testing result of whole grain vs. milled extraction of 59 rice samples in respect to their residue analysis classification

Rice sample No.	Inhibited fraction of AChE activity[z]					
	Whole grain extraction			Milled rice extraction		
	Group I	Group II	Group III	Group I	Group II	Group III
1	0.0000	0.0000	0.3500	0.0000	0.0000	0.2600
2	0.0000	0.0000	0.4700	0.0000	0.0000	0.2600
3	0.0000	0.0000	0.8400	0.0000	0.4500	0.8200
4	0.0000	0.1700	0.7100	0.0000	0.4900	0.5800
5	0.0000	0.4800	0.7000	0.0000	0.5200	0.6500
6	0.0000	0.5400	0.5400	0.0000	0.5400	0.6100
7	0.0000	0.6300		0.0000	0.5500	
8	0.0000	0.4700		0.0000	0.4400	
9	0.0000	0.1400		0.0000	0.3200	
10	0.0000	0.2000		0.0000	0.2000	
11	0.0000	0.1200		0.0000	0.4000	
12	0.0000	0.4300		0.0000	0.4800	
13	0.0000	0.3100		0.0000	0.4100	
14	0.0000	0.5200		0.3900	0.3900	
15	0.0000	0.1900		0.0200	0.2100	
16	0.0000	0.1200		0.3200	0.2600	
17	0.0000	0.2000		0.0600	0.2200	
18	0.1600	0.1600		0.1800	0.0600	
19	0.1500	0.3800		0.0900	0.2500	
20	0.3200	0.2000		0.0600	0.2400	
21	0.1400	0.2900		0.1000	0.3800	
22	0.3100	0.1800		0.0900	0.2200	
23	0.0400	0.2200		0.1000	0.0800	
24	0.1700	0.3600		0.4900	0.1200	
25	0.3900	0.0800		0.4200	0.0300	
26		0.2400			0.1600	
27		0.3100			0.2500	
28		0.8000			0.7500	
Samples (N)	25	28	6	25	28	6
Mean	0.0672	0.2764	0.6017	0.0928	0.3007	0.5300
S. E.	0.0237	0.0373	0.0736	0.0298	0.3583	0.0919
95% confidence ranges						
Lower limit	0.0184	0.1999	0.4124	0.0313	0.2272	0.2938
Upper limit	0.1160	0.3529	0.7910	0.1543	0.3742	0.7662

[z] Group I: residues are not detectable (ND); Group II: OP and Carb residues are detected but below the tolerance; Group III: OP and Carb residues are detected and violate the tolerance.

Table 2. The cross examination of 59 rice samples by the toxicity test and chemical analysis

AChE inhibiton	No. of samples[z]			Subtotal
	Group I (N=25)	Group II (N=28)	Group III (N=6)	
Whole grain extraction				
0~27.6%	22	16	-	38
27.6~60.2%	3	10	3	16
60.2% and above	-	2	3	5
Milled rice extraction				
0~30.1%	21	15	2	38
30.1~53.0%	4	10	-	14
53.0% and above	-	3	4	7

[z] Group I: residues are not detectable (ND); Group II: OP and Carb residues are detected but below the tolerance; Group III: OP and Carb residues are detected and violate the tolerance.

The results clearly indicated that OP and Carb insecticide residues in rice grain can be detected by the AChE inhibition test and the results are quantitatively similar to chemical analysis. This makes it possible to establish a rapid analysis of insecticide residue by AChE inhibition test. AChE inhibition was also specific to OP and Carb residues and not affected by the plant material. This we construe from the fact that in whole grain extraction test, 20 samples out of 59 showed no AChE inhibition. Seventeen of these samples belonged to group I by chemical analysis, and only 3, or barely 11%, to group II, and none to group III. This implies that the AChE inhibition comes from OP and Carb residues only and not from the plant material in rice grain. Besides the lower limit of AChE inhibition of group I samples was set very low, 1.8%, or close to zero, even then no interference from plant material was detected. Furthermore, the AChE inhibition positive samples were subjected to confirmation by TLC, which further ruled out the possibility of false positive (Fig. 2).

Inevitably, a discriminating level of AChE inhibition has to be established when one screens large number of rice samples for residues of OP and Carb insecticides. The advantages and disadvantages of setting discriminating levels of toxicity should be evaluated to arrive at optimal decision. The 95% confidence range of group III samples by whole grain extraction procedure ranged from 41 to 79 % AChE inhibition. The outcome of stepwise 10% increment in AChE inhibition on various rejection thresholds for 59 samples is presented in Table 3. The major concern is the detection rate of group III samples. By establishing the discriminating level at 60% AChE inhibition, the detection rate is 50%. While gradually lowering the discriminating level by 10 % interval, the detection rate will increase to 66.7% and finally 83.3% at 50% and 40% AChE inhibition, respectively. At these levels, the false negative rate drops from 5.5% to 3.9% to 2.1%, respectively. However, possible unnecessary rejection rate increases from 40% to 50% to 58%, respectively.

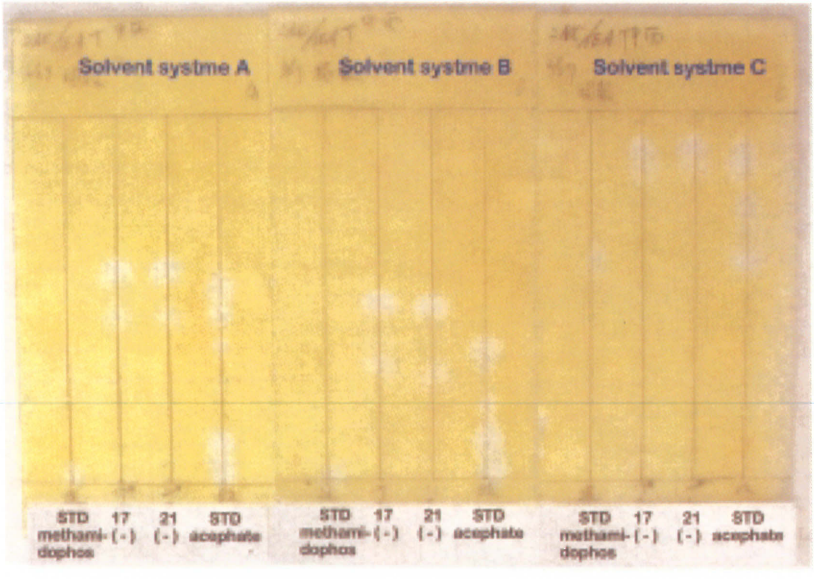

Fig. 2. Thin layer chromatography analysis of rice sample with positive acetylcholinesterase inhibition by three solvent developing systems.

DISCUSSION

The certification of safety inspection for food commodities is a very common matter in now-a-days. In rice production, two large groups of neurotoxic insecticides groups, OP and Carb, which are used widely, are cause of concern over their safety. Before these insecticides have been removed from agricultural usage, inspection of their residues or neurotoxicities is needed. Implementation of RBPR by TARI in 1985 and the favorable response it received from the consumers have resulted in establishment of more than 120 RBPR stations to inspect safety of fruits and vegetables on this island. The TARI RBPR has impacted even internationally in countries like Korea, Vietnam, Thailand, and Mainland China, where similar fruits and vegetable safety inspection have been adopted. Although fruits and vegetables in general receive more frequent applications of pesticides than cereals, considering larger quantities of their production and consumption, food safety inspection of these staple food commodities is also needed. Since chemical analysis of residues can offer information well after harvesting and in some case even marketing, a simpler, quicker and accurate toxicity test can fill the gap between harvest and the information from chemical analysis.

To expand RBPR for detection of hazardous pesticide residues in cereals is a new challenge. Utilizing the rice samples with known residue status, we have detected residues of OP and Carb insecticides by AChE inhibition test that matches fairly with the results obtained by chemical analysis. This will make it possible to establish rapid residue screening method for rice and other cereals. The naturally occurring chemicals in rice grain do not cause any interference in AChE inhibition, as evidenced by the absence of false positive in the test. The AChE inhibition observed in rice sample was thus exclusively due to the presence of pesticide residues in rice grains.

Statistical analysis demonstrated that AChE test is useful in classifying rice samples into low, medium, and high risk categories and it conformed with the results of chemical analysis. Hence the idea of implementing RBPR for rice safety inspection should receive consideration. However, the key criterion is how to set the toxicity discriminating level for screening large number of samples. If the set level is 60%, samples with high residue contents that might violate tolerance will pass the test without being detected,

Table 3. Different toxicity discriminating levels and risk assessment for 59 rice samples in respect to their residue analysis results

AChE inhibition range (%)	No. of samples[z]			Subtotal	Risk assessment		
	Group I	Group II	Group III		Detection rate (%)[y]	Unnecessay rejection (%)[x]	False negative (%)[w]
0-60	25	26	3	54			
>60	0	2	3	5	3/6 (50.0)	2/5 (40.0)	3/54 (5.5)
0-50	25	24	2	51			
>50	0	4	4	8	4/6 (66.7)	4/8 (50.0)	2/51 (3.9)
0-40	25	21	1	47			
>40	0	7	5	12	5/6 (83.3)	7/12 (58.0)	1/47 (2.1)

[z] Group I: residues are not detectable (ND);Group II: OP and Carb residues are detected but below the tolerance; Group III: OP and Carb residues are detected and violate the tolerance.

[y] No. of samples with toxicity over the discriminating level / No. of group III samples.

[x] No. of samples contained residues below the tolerance /No. of samples with toxicity over the discriminating level.

[w] No. of samples contained residues over the tolerance /No. of samples with toxicity lower the discriminating level.

which is harmful to consumers. However, if the level is set lower to 40%, many samples with tolerable level of residues will be rejected, which is unfair to the producers.

We have listed advantages and disadvantages of setting different discriminating toxicity levels and the risk assessment parameters in Table 3. These parameters, especially the detection rate for violations, the unnecessary rejection rate, and the false negative rate should be useful in making decision in setting optimum discriminating level. Based on the information generated by us, whether RBPR can be used in rice and setting up of discriminating level that is acceptable by both consumer and producer, need to be opened for public discussion and decision.

The results of this study have confirmed three important points. First, the residues of OP and Carb in rice can be detected by the AChE inhibition test without the interference of plant material. Second, the RBPR is sensitive enough to detect the anticholinergic residues to a level corresponding to the national tolerance stand for the two groups of chemicals. This should make it possible to use RBPR to categorize the grain samples into low, medium, and high risk groups for the presence of neurotoxic residues. Third, the AChE is subjected to inhibition by OP and Carb residues only, which results in the measurement of sum of all anticholinergic pesticide residues present in a particular sample. The total toxicity thus measured conforms with recent updated tolerance regulation of FQPA law of the United States.

It must be emphasized that RBPR still is a measurement of toxicity, which may not be able to replace the chemical analysis of insecticide residues, particularly when there is need to trace the insecticide quantitatively and qualitatively. Nevertheless, use of RBPR to evaluate the safety of rice is definitely feasible in many ways. For examples, the test can be used to screen the organic rice because no pesticide is supposed to be found in those products. Secondly, the test is convenient for evaluating safety of rice produced in different areas, different seasons, and different years. Finally, RBPR can be used in indexing the safety of rice for the improvement of insecticide usage. Because RBPR is a reliable, economical, and easy to use, it is convenient and useful tool to improve the safety of rice production for better consumer protection.

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稻米含抗膽鹼激性物質之快速檢定研究與應用

高靜華、曾佳琳、鄭允

摘　要

高靜華、曾佳琳、鄭允。2003。稻米含抗膽鹼激性物質之快速檢定研究與應用。中華農業研究 52:324-333。

稻米爲東方人的主食，栽培時常需使用農藥以減少病蟲害，其中以在稻米上登記逾 40 種之氨基甲酸鹽及有機磷劑的殘毒最值得重視。此兩類殺蟲劑的毒理機制相同，均作用於動物神經系統中負責傳導的乙醯膽鹼酯酶(AChE)，其致毒性有累加作用。本研究係嘗試以檢驗蔬果農藥殘毒之生化技術，發展稻米的殘毒快速檢測法，試驗材料爲 59 件已具農藥殘留化學分析資料之稻米樣本，依無檢出、檢出未超量及超量違規三類分析結果，樣品各有 25、28 及 6 件。所有樣品均進行全粒及打碎兩種萃取處理，再個別以生化法檢驗之。三類樣品之全粒萃取檢驗結果顯示，乙醯膽鹼酯酶抑制值不僅與化學分析結果成正相關，且不同類別之統計差異極顯著。打碎處理的結果大致與全粒者相同，惟第三類樣品中有 2 件之抗乙醯膽鹼酯酶反應值下降，造成第三類與第二類樣品間的 95%可信度範圍發生重疊，而差異不顯著。初步結果顯示，以高感度的乙醯膽鹼酯酶快速檢測稻米中所含抗膽鹼物質(即氨基甲酸鹽及有機磷劑之殘毒)時，全粒萃取的檢驗可信度甚高，測試中也確認稻穀不含干擾生化檢驗成分。快速檢驗採用不同檢出標準之風險評估顯示，檢出標準由 60%抑制率調降爲 50%及 40%時，違規樣品的檢出率由 50%上升至 66.7%及 88.3%，合格樣品之僞陰性由 5.5%下降至 3.9%及 2.1%，檢出樣品中仍低於容許量者則由 40%上升至 50%及 58%。稻米殘毒的快速檢驗並非用以取代現行之檢驗法，但對於提昇稻米品質安全有諸多用途，如：(1)用於辨別有機米之眞僞；(2)評估不同地區、不同期作或不同年度稻米之相對安全性；(3)可提供多種農藥殘留時之總毒性數據，符合美國最新 FQPA 法案之要求；(4)能在有限經費下迅速檢驗成百上千件樣品。利用以上優點可全面評鑑全國水稻之安全品質，透視並改進水稻用藥問題。

關鍵詞：農藥殘毒快速檢驗、稻米、有機磷劑、氨基甲酸鹽劑、乙醯膽鹼酯酶。

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1. 行政院農業委員會農業試驗所研究報告第 2173 號。接受日期:92 年 10 月 1 日。

2. 本所應用動物組副研究員、計畫助理及研究員。臺灣省　臺中縣　霧峰鄉。

3. 通訊作者，電子郵件: chkao@wufeng.tari.gov.tw ; 傳眞機: (04)23309097。